Posttranslational Modification of the Carboxy-Terminal Region of the β Subunit of Human Chorionic Gonadotropin†

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ABSTRACT: The β subunit of human chorionic gonadotropin (hCG) contains at its carboxy terminus an extension of 29 amino acids not found in the β subunits of the other glycoprotein hormones. This region provides the sites of attachment of four serine-linked oligosaccharide chains. We have examined the synthesis of this subunit in a cell-free translation system derived from Krebs II ascites tumor cells. The primary translation product was found to undergo a temperature-dependent posttranslational modification which resulted in an increase in apparent molecular weight of 2000 on sodium dodecyl sulfate gel electrophoresis. This modification was specific for the β subunit of hCG, since no changes were observed for the β subunit of bovine luteinizing hormone or for the α subunits of either hormone. The increase in molecular weight occurred in the absence of microsomal membranes and was not due to the addition of N-linked carbohydrate. An identical shift was observed when pre-hCG β was incubated with extracts of human placenta. The site of modification was localized by fingerprint analysis to a carboxy-terminal tryptic peptide which contains two of the four O-glycosylated serine residues in the mature form of the subunit. The modified protein was resistant to oligosaccharidase digestion and β-elimination, indicating that it does not contain O-linked oligosaccharides of the type found on mature hCG β. These results demonstrate that a specific modification of the carboxy-terminal segment of hCG β synthesized in vitro occurs in the absence of O-linked glycosylation.

The specificity of action of the glycoprotein hormones, which share a common α subunit, is largely determined by their individual β subunits (Pierce & Parsons, 1981). The β subunit of the placental hormone human chorionic gonadotropin (hCG)[1] stands out from those of luteinizing hormone (LH), follicle-stimulating hormone, and thyroid-stimulating hormone by the presence of an extension of 29 amino acids at its carboxy terminus (Birken & Canfield, 1980). This unique extension provides the sites of attachment of the four serine-linked oligosaccharide moieties present on the mature form of hCG secreted by human trophoblast cells (Birken & Canfield, 1977; Keutmann & Williams, 1977).

Recent evidence has suggested that an additional form of hCG β may be secreted by some tumor cells (Cole et al., 1982; Ruddon et al., 1983). This material is immunoprecipitated by hCG β specific antiserum but is thought to be missing or to contain an altered carboxy-terminal region (Cole et al., 1982). Multiple immunoreactive species of hCG β have also been observed in cell-free translation systems containing first-trimester placental mRNA (Boime et al., 1982; Godine et al., 1982). However, because of the lack of biochemical characterization of these products, their significance is unclear.

The mechanism by which carbohydrate is added to the carboxy-terminal region of hCG β is not understood. Pulse–chase studies in JAr (Ruddon et al., 1980, 1981) and BeWo (Hanover et al., 1982) choriocarcinoma cells have suggested that O-glycosidically linked carbohydrate is transferred to hCG β just prior to secretion. Glycosyltransferases thought to be responsible for the O-linked glycosylation of submaxillary and intestinal mucins have been identified in Golgi membrane preparations (Kim et al., 1971; Andersson & Eriksson, 1981). It is generally believed that the Golgi is also the site of O-glycosylation of other glycoproteins (Hanover et al., 1980; Johnson & Spear, 1983), although at least one report suggested that the initial event may occur during translation (Strous, 1979). A recent study (Cummings et al., 1983) has shown that O-linked *N*-acetylgalactosamine is added to the LDL receptor precursor prior to the action of α-mannosidase I, an enzyme known to be localized in the early (cis) Golgi region (Goldberg & Kornfeld, 1983).

We have used reconstituted ascites tumor lysates to follow the processing of glycoprotein translation products by endoplasmic reticulum and Golgi-derived microsomes (Bielinska & Boime, 1978). In studies designed to examine the initial processing events related to O-glycosylation in this cell-free system, we observed that the β subunit of hCG underwent a temperature-dependent posttranslational modification. This modification was specific for hCG β since no changes were observed for the LH β translation product. Furthermore, we show that the site of this modification is the carboxyl-terminal extension of the hCG β subunit and that the reaction occurs without addition of O-linked carbohydrate.

Experimental Procedures

Materials. [^{35}S]Methionine (>600 Ci/mmol), [^{35}S]cysteine (>600 Ci/mmol), [^{3}H]leucine (140 Ci/mmol), and [^{3}H]alanine (75 Ci/mmol) were purchased from New England Nuclear. [^{3}H]Proline (114 Ci/mmol), [^{3}H]serine (13 Ci/mmol), [α-^{32}P]ATP (3000 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham-Searle. HPLC-grade acetonitrile and phosphoric acid were purchased from Fisher,

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[1] Abbreviations: hCG, human chorionic gonadotropin; LH, luteinizing hormone; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; S-100, ribosome-free supernatant; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; NRS, normal rabbit serum.

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and trifluoroacetic acid was from Pierce Chemical Co. Acetic acid was from Mallinckrodt. Guanidine thiocyanate was obtained from Fluka, CsCl was a product a Varlacoid Chemical Co., cycloheximide was purchased from Sigma Chemical Co., and bovine pancreatic ribonuclease was from Boehringer Mannheim.

Preparation of RNA. First-trimester human placental RNA was isolated by phenol extraction as previously described (Szczesna & Boime, 1976) or by a modification of the method of Glisin et al. (1974). In the latter procedure, pooled first-trimester placental tissue was homogenized in 2 volumes of a solution containing 4 M guanidine thiocyanate, 1 M β-mercaptoethanol, 50 mM sodium acetate (pH 5.2), and 1 mM EDTA by using a motor-driven homogenizer equipped with a Teflon pestle. Tissue fragments were removed by centrifugation at 8000*g* for 5 min, and 1.0 g of CsCl was added per 2.5 mL of supernatant. This solution was layered onto a 5.7 M CsCl cushion and centrifuged at 100000*g* for 20 h. The resulting RNA pellet was dissolved in water, made to 0.3 M sodium acetate (pH 5.2), and precipitated with 2 volumes of 95% ethanol. The pellet was ethanol precipitated a second time and brought to a final concentration of 1.75 mg/mL in sterile water. Bovine pituitary RNA was prepared as previously described (Keller et al., 1980).

Analysis of Cell-Free Translation Products. Ascites ribosomes, membranes, and ribosome-free supernatant (S-100) were prepared as described (Szczesna & Boime, 1976). Translations were performed in 50-μL reaction mixtures containing 1.5 mM ATP, 0.3 mM GTP, 15 mM creatine phosphate, 0.16 mg/mL creatine kinase (Boehringer Mannheim), 1.8 mM magnesium acetate, 66 mM KCl, 15 mM HEPES (pH 7.6), 40 μM each of the nonradioactive amino acids, 2.5 mM dithiothreitol, 0.4 mM spermidine, 10 μL of ascites S-100, 3 μL of ribosomes, and 5–6 μg of RNA. Translation products were immunoprecipitated by using antisera directed against the α and β subunits of hCG (Daniels-McQueen et al., 1978), or of ovine LH (provided by Dr. Tom Landefeld, The University of Michigan), as previously described (Fetherston & Boime, 1982) except that an initial cycle of immunoprecipitation using normal rabbit serum was included to reduce nonspecific background. Labeled products were analyzed on 20% polyacrylamide gels containing 0.1% SDS (Szczesna & Boime, 1976) and detected by fluorography (Chamberlain, 1979).

Acetone Precipitation. Immunoprecipitates were solubilized by boiling for 5 min in a buffer containing 1% SDS, 1.5% Triton X-100, 100 mM β-mercaptoethanol, and 20 mM Tris-HCl (pH 7.0). Five milligrams of bovine serum albumin was added and the protein precipitated by the addition of 20 volumes of ice-cold acetone (Hortin & Boime, 1981). The precipitate was collected by centrifugation at 8000*g* for 10 min and lyophilized.

Automated Edman Degradation. Amino-terminal sequence analysis of acetone-precipitated translation products was performed in an updated Beckman 890A sequencer as previously described (Birken et al., 1981).

Tryptic Fingerprint Analysis. Translation reactions containing first-trimester placental RNA were scaled up 40-fold and immunoprecipitated with hCG β antiserum. The products were collected by acetone precipitation and subjected to reduction and carboxymethylation as described (Morgan et al., 1975). Alkylated materials were separated on Sephadex G-25 columns equilibrated in 50% acetic acid and lyophilized in preparation for tryptic digestion. A 10 mg/mL solution of TPCK-treated trypsin (Worthington, 180 units/mg) in 1 mM HCl (pH 3) was used in all digestions. Samples were dissolved in 2.0 mL of 0.3 M ammonium bicarbonate, and 20 μL of trypsin was added. After 45 min of incubation at room temperature, an additional 10 μL of trypsin was added and the incubation continued for another 45 min. At this time, either the reaction was stopped by lyophilization (limited digestion) or another 10 μL of trypsin was added and the reaction allowed to continue for 18 h (complete digestion). The digested material was dissolved in 200 μL of 0.1% trifluoroacetic acid and filtered through 0.2-μm cellulose acetate centrifugal microfilters (Bioanalytical Systems). Peptides were separated by reverse-phase HPLC using Altex 110A pumps and an Axxiom 710 controller (P. J. Cobert, St. Louis, MO). Samples were loaded onto a Whatman Protesil 300 octyl column and eluted with a 70-min linear gradient of 0–40% acetonitrile at a flow rate of 1 mL/min. One-milliliter fractions were collected and counted in 5 mL of 3a70B complete counting cocktail (Research Products International). Tryptic peptide standards were generated from synthetic peptide analogues of hCG β identical in amino acid sequence with residues 123–145 [a gift of Matsuura and Chen (Matsuura et al., 1978)] or residues 111–145 (obtained from the Contraceptive Development Branch, Center for Population Research, NICHD, Bethesda, MD). These synthetic peptides were partially digested with trypsin and chromatographed on the reverse-phase HPLC system. The elution positions of tryptic peptides 115–122, 123–133, 134–145, and 123–145 were confirmed by amino acid analysis.

Anion-Exchange HPLC. Reverse-phase fractions corresponding to tryptic peptides 123–133 were pooled and loaded onto an Altex Ultrasil-Ax anion-exchange column equilibrated with 22 mM sodium phosphate (pH 6.5). The column was washed with 10 mL of the loading buffer and eluted with a 50-min linear gradient from 0 to 50% acetic acid in 22 mM sodium phosphate at a flow rate of 1 mL/min (Baldwin et al., 1983). One-minute fractions were collected.

Glycosidase Treatment. Translation reaction mixtures were incubated with 1.0 milliunit of neuraminidase (Sigma) and 1.2 milliunits of α-*N*-acetyl-D-galactosamine oligosaccharidase from *Clostridium perfringens* (Bethesda Research Laboratories) in 0.5 mL of 50 mM sodium acetate (pH 5.5) for 18 h at 37 °C. Samples were neutralized by the addition of 0.2 M NaOH, immunoprecipitated, and analyzed by SDS electrophoresis. Control experiments indicated that this treatment removed both O-linked and N-linked oligosaccharides from mature hCG β subunit.

Alkaline β-Elimination. Translation products labeled with ^{3}H in the 3-position of serine residues were immunoprecipitated with hCG β antiserum and acetone precipitated. The acetone pellet was incubated for 48 h at 45 °C in 200 μL of 0.1 M NaOH and 0.3 M sodium borohydride according to the procedure of Baenziger & Kornfeld (1974). This treatment releases O-glycosidically linked oligosaccharides and converts the linkage serine residues to alanine (Downs & Pigman, 1976). The reaction was stopped by the addition of 300 μL of 0.4 M HCl, and the samples were hydrolyzed in 6 N HCl (110 °C, 20 h) and analyzed on a Waters programmable HPLC amino acid analysis system equipped with postcolumn *o*-phthalaldehyde detection. The ^{3}H radioactivity of collected fractions was compared to elution positions of serine and alanine standards.

Phosphoprotein Phosphatase Treatment. Rabbit muscle phosphoprotein phosphatase and ^{32}P-labeled glycogen phosphorylase were generously provided by Dr. Balwant S. Khatra (Vanderbilt University). Acetone-precipitated ^{35}S-labeled hCG

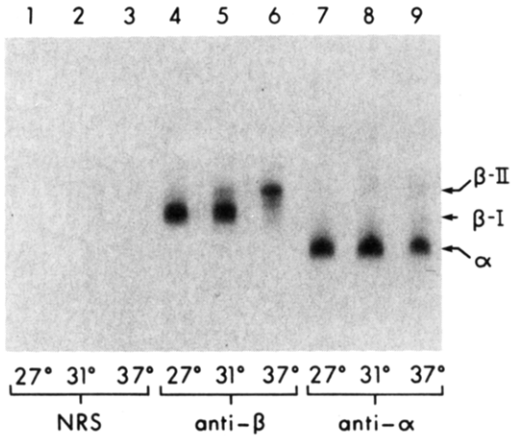

FIGURE 1: Effect of temperature on cell-free translation of hCG subunits. Ascites lysates containing placental RNA and [^{35}S]cysteine were incubated for 2 h at 27 °C (lanes 1, 4, and 7), 31 °C (lanes 2, 5, and 8), or 37 °C (lanes 3, 6, and 9). Translation products were immunoprecipitated with normal rabbit serum (NRS) (lanes 1–3), hCG β antiserum (lanes 4–6), or hCG α antiserum (lanes 7–9) and electrophoresed on SDS–polyacrylamide gels. β-I and β-II indicate low and high molecular weight forms of hCG β, respectively. α indicates hCG α.

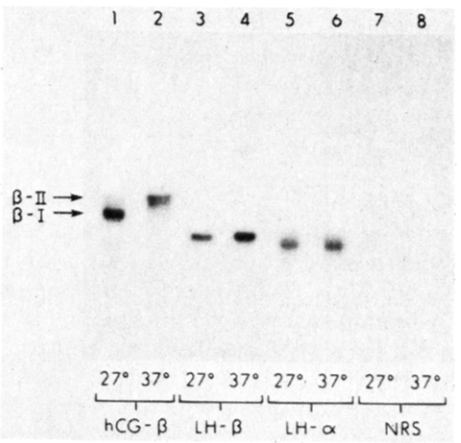

FIGURE 2: Effect of temperature on cell-free translation of LH subunits. Ascites lysates containing [^{35}S]cysteine and either placental RNA (lanes 1 and 2) or bovine pituitary RNA (lanes 3–8) were incubated for 2 h at 27 °C (lanes 1, 3, 5, and 7) or 37 °C (lanes 2, 4, 6, and 8). Translation products were immunoprecipitated with hCG β antiserum (lanes 1 and 2), LH β antiserum (lanes 3 and 4), LH α antiserum (lanes 5 and 6), or normal rabbit serum (lanes 7 and 8) and subjected to SDS gel electrophoresis.

β translation products (20 000 cpm) were incubated at 30 °C for various times in 50 μL of an assay mixture containing 50 mM Tris (pH 7.5), 1 mM dithiothreitol, and the phosphatase (Swarup et al., 1981). The labeled protein was precipitated in 10% trichloroacetic acid and analyzed on SDS electrophoresis. The ^{32}P-labeled phosphorylase served as an internal positive control in all treatments.

Results

Human first-trimester placental RNA was translated in reconstituted Krebs II ascites tumor lysates containing [^{35}S]cysteine. No microsomal membranes were added. HCG α and β translation products were immunoprecipitated by subunit-specific antisera and analyzed by SDS gel electrophoresis. When these lysates were incubated for 2 h at 31 °C, hCG β antiserum immunoprecipitated two bands (Figure 1, lane 5). The major product, termed β-I, migrated with an apparent molecular weight of 18 000. This species corresponds to the pre-form of the β subunit that contains a signal peptide (Birken et al., 1981). Also synthesized was a small amount of a slightly larger protein with an apparent molecular weight of 20 000 (β-II). When first-trimester RNA was translated in lysates incubated at 37 °C, however, β-II was the predominant form detected (lane 6). In contrast, only β-I was synthesized at 27 °C (lane 4). Regardless of the temperature of incubation, neither protein was precipitated from reaction mixtures by normal rabbit serum (lanes 1–3).

The temperature dependence appeared to be specific for hCG β since only one form of the hCG α subunit was synthesized in lysates incubated at all three temperatures (Figure 1, lanes 7–9). To further examine the specificity of this effect, RNA isolated from bovine pituitary was translated at 27 and 37 °C, and the products of the reaction were immunoprecipitated with antisera specific for the α and β subunits of LH (Figure 2). Only single forms of pre-LH β (lanes 3 and 4) and pre-LH α (lanes 5 and 6) were detected. Thus, the temperature effect was specific only for the hCG β subunit.

Because these incubations were performed in the absence of microsomal membranes, cleavage of the signal peptide should not have occurred (Szczesna & Boime, 1976). To verify that the heterogeneity observed was not due to different signal peptides, partial amino-terminal sequencing was performed. β-I and β-II were synthesized in the presence of [^{35}S]methionine and [^{3}H]leucine, isolated from SDS gels, and subjected to automated Edman degradation. Methionine residues were detected at cycles 1, 3, and 15 in both proteins, as was a string of leucine residues at cycles 7–13 (Figure 3). These positions correspond exactly with the known sequence of the amino terminus of the pre-hCG β subunit (Birken et al., 1981; Fiddes & Goodman, 1980). Furthermore, the ^{3}H counts detected at cycle 25 in both samples represent leucine at position 5 of the mature subunit. These results demonstrate that β-I and β-II have identical amino termini which correspond in sequence and length with the known hCG β signal peptide.

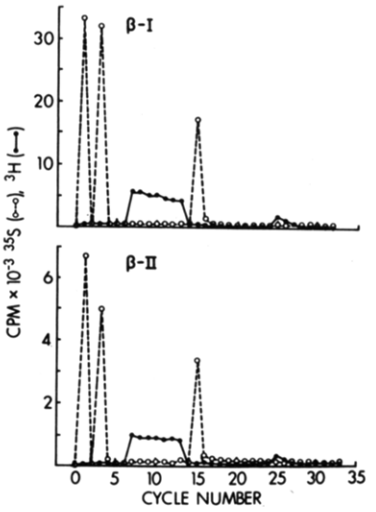

FIGURE 3: Partial amino-terminal sequence analysis of β-I and β-II. hCG β translation products were immunoprecipitated from reactions containing [^{35}S]methionine and [^{3}H]leucine, collected by acetone precipitation as described under Experimental Procedures, and subjected to automated Edman degradation. Upper panel, hCG β-I isolated from reaction mixtures incubated at 27 °C; lower panel, hCG β-II isolated from reaction mixtures incubated at 37 °C.

Posttranslational Conversion of β-I to β-II. The above data suggested that β-I and β-II may be translated from the same mRNA but differ due to posttranslational modification. Alternatively, the two bands could have resulted from the translation of two different mRNAs, each with the same amino-terminal sequence, but differing in distal coding regions.

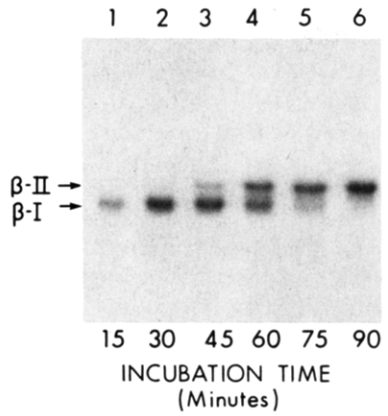

FIGURE 4: Time course of cell-free translation of hCG β subunit. Ascites lysates containing placental RNA and [^{35}S]cysteine were incubated at 37 °C for various times, immunoprecipitated with hCG β antiserum, and subjected to SDS gel electrophoresis.

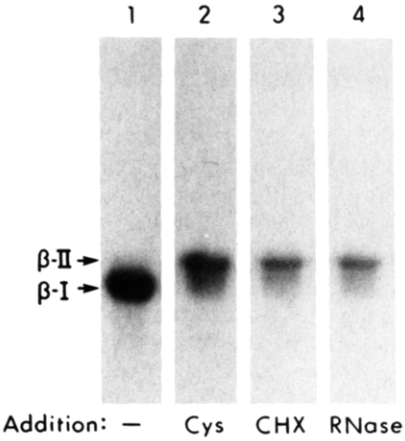

FIGURE 5: Posttranslational conversion of β-I to β-II. Ascites lysates containing placental RNA and [^{35}S]cysteine were incubated for 1 h at 27 °C. Aliquots were either immunoprecipitated directly (lane 1) or incubated for 1 h at 37 °C in the presence of 60 μM unlabeled cysteine (lane 2), 2 mM cycloheximide (land 3), or 100 μg/mL bovine pancreatic ribonuclease (lane 4). Samples were immunoprecipitated with anti-hCG β antiserum and subjected to SDS gel electrophoresis.

To help distinguish between these possibilities, the time course of formation of β-I and β-II at 37 °C was examined (Figure 4). β-I was seen within 15 min of incubation (lane 1), attained a peak after 30–45 min (lanes 2 and 3), and declined thereafter (lanes 4–6). In contrast, β-II was first detected only after 45 min of incubation (lane 3) and by 90 min became the major product (lane 6). This experiment suggested that the initial translation product was β-I, which shifted to the higher molecular weight form upon longer incubation at 37 °C. Partial conversion also occurred at 27 °C, but at a much reduced rate (not shown).

Conclusive evidence that β-II did arise as a result of posttranslational modification of β-I was obtained by pulse–chase experiments (Figure 5). Lysates were first incubated for 60 min at 27 °C, producing β-I (lane 1). This material was then incubated for an additional 60 min at 37 °C in the presence of excess unlabeled cysteine, cycloheximide (CHX), or pancreatic ribonuclease (lanes 2–4). In all cases, β-I was chased into β-II. The failure of cycloheximide or ribonuclease to inhibit this conversion indicated that the production of β-II at 37 °C was not due to selective utilization of a different mRNA or to readthrough translation. Instead, β-II resulted from posttranslational modification of β-I.

The posttranslational nature of this modification permitted us to develop an assay in which the conversion of β-I to β-II

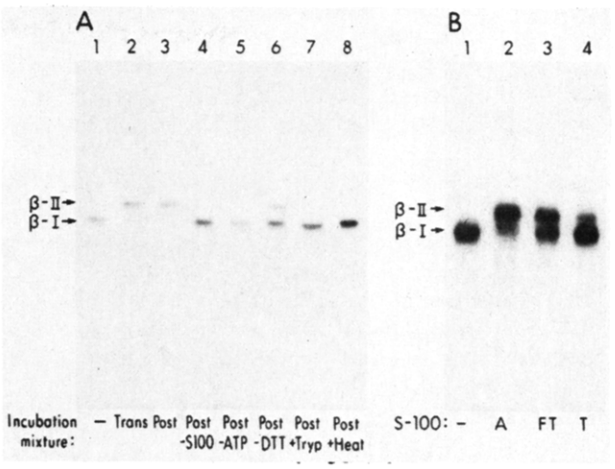

FIGURE 6: Conversion of immunoprecipitated β-I to β-II; dependence on components of the translation reaction mixture. (A) [^{35}S]Cysteine-labeled β-I was immunoprecipitated from translations scaled up 20-fold and incubated for 1 h at 27 °C. The protein was collected by acetone precipitation, and aliquots containing 20 000 cpm were either analyzed directly (lane 1) or incubated for 5 h at 37 °C (lanes 2–8). Lane 2, incubation at 37 °C was performed in the presence of the total translation reaction mixture (Trans) described under Experimental Procedures; lane 3, incubation was performed in the presence of a posttranslational reaction mixture (Post) consisting of 1.5 mM ATP, 2.5 mM dithiothreitol (DTT), 1.8 mM Mg^{2+}, 43 mM K^+, and ascites S-100 only. Lanes 4, 5, and 6 were identical with lane 3 except that S-100, ATP, or dithiothreitol was omitted, respectively. Lanes 7 and 8 contained ascites S-100 which had been pretreated with 20 μg of trypsin for 30 min at 31 °C or incubated at 56 °C for 10 min, respectively. (B) In a separate experiment, 10 μL of S-100 prepared from human first-trimester (FT, lane 3) or term (T, lane 4) placenta was substituted for ascites S-100. Lane 1, no incubation; lane 2, 10 μL of ascites S-100 (A).

was uncoupled from protein synthesis. [^{35}S]Cysteine-labeled β-I was immunoprecipitated from translation mixtures incubated at 27 °C and acetone precipitated as described under Experimental Procedures. The acetone powder was incubated with fresh ascites lysate for 5 h at 37 °C, converting β-I to β-II (Figure 6, lanes 1 and 2). The conditions of the second incubation did not need to be compatible with translation and therefore could be varied independently of the first incubation.

Using this assay, it was found that, of the components normally present in the translation reaction mixture, only ascites S-100, 1.5 mM ATP, and 2.5 mM dithiothreitol (DTT) were necessary to support the posttranslational conversion (Figure 6A, lane 3). Removal of S-100 or ATP from the incubation completely prevented the modification (lanes 4 and 5), and elimination of DTT partially inhibited it (lane 6). In addition, S-100 which had been digested with trypsin (lane 7) or heat treated (56 °C, 10 min) prior to the incubation (lane 8) failed to convert β-I to β-II, indicating that the reaction is dependent upon a heat-labile enzymatic activity.

To determine whether this reaction was unique to ascites tumor extracts, S-100 preparations from human placenta were tested in the posttranslational assay (Figure 6B). First-trimester placental S-100 produced a conversion of β-I to β-II similar in magnitude to that seen with ascites S-100 (Figure 6B, lanes 2 and 3). Extracts of term placenta were less efficient than ascites (lane 4). This result demonstrates that the activity is present in tissue which normally synthesizes hCG.

Tryptic Fingerprint Analysis. To identify which regions of β-II were modified, tryptic fingerprint analysis was performed. First-trimester RNA was translated at 27 or 37 °C in the presence of several different radiolabeled amino acids. The two forms of the subunit were then isolated and digested with trypsin, and the resulting peptides were analyzed on

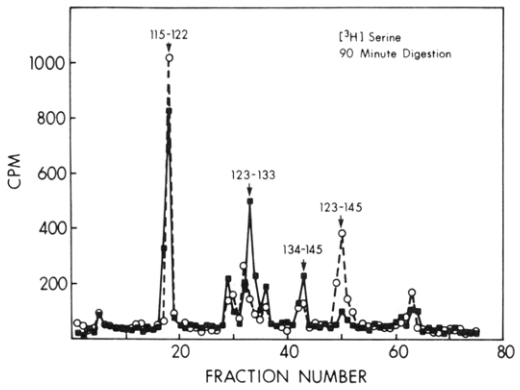

FIGURE 7: Tryptic fingerprint maps of β-I and β-II labeled with [3H]serine. β-I and β-II were immunoprecipitated from translation mixtures containing [3H]serine and collected by acetone precipitation. The proteins were reduced and carboxymethylated and then digested with trypsin for 90 min at 23 °C. The resulting tryptic peptides were separated by reverse-phase HPLC on a Whatman Protesil 300 octyl column. The peptides were eluted at 1 mL/min with a 70-min linear gradient of 0–40% acetonitrile in 0.1% trifluoroacetic acid and collected in 1-mL fractions. (■) β-I; (○) β-II. Arrows indicate the elution positions of synthetic peptide standards identical in sequence with hCG β residues 115–122, 123–133, 134–145, and 123–145.

reverse-phase HPLC. The overall pattern of peaks was consistent in several experiments. In every case, the only peptides that differed between the two forms corresponded with sequences from the carboxy-terminal region of hCG β.

Figure 7 shows the [3H]serine-containing peptides generated in a 90-min tryptic digestion. A large peak which coeluted with a synthetic peptide identical in sequence with residues 123–145 of hCG β was present in the β-II digest but not in the β-I digest. In addition, decreased quantities of radioactivity coeluting with synthetic peptides 123–133 and 134–145 were recovered from β-II. The elution position of fragment 115–122, which contains four serine residues (see Figure 10), did not differ between β-I and β-II. These results suggested that arginine-133 in the modified protein was resistant to tryptic digestion. Similar experiments using [3H]proline- and [3H]alanine-labeled samples supported this interpretation (Figure 8). Proline-labeled peaks coeluted with peptides 123–133, 134–145, and 123–145 (Figure 8A), consistent with the large number of proline residues in these fragments (Figure 10). The only alanine residue present in the carboxy-terminal region of hCG β is located at position 123 (Figure 10). The recovery of labeled alanine eluting with synthetic peptides 123–133 and 123–145, but not with synthetic peptide 134–145 (Figure 8B), confirmed the identification of these peptides in the β-I and β-II digests. The tryptic site at arginine-133 was consistently more resistant to cleavage in β-II than in β-I (Figure 8A,B).

The selective resistance of β-II to tryptic digestion suggested that modified amino acids may be present on peptide 123–145, presumably near the arginine-133 cleavage site. This modification did not completely block the action of trypsin at this site, however, since in more extensive (18-h) digestions cleavage of β-II did occur (Figure 8C). In this case, one of the two resulting tryptic peptides, 123–133, consistently eluted two to three fractions earlier in β-II digests than in β-I digests. This pattern of elution was retained when the peaks corresponding to this peptide were collected from β-I and β-II digests and rerun on reverse-phase HPLC (Figure 8D). The other cleavage product, peptide 134–145, did not significantly differ from either the corresponding β-I peak or the synthetic standard. The slight differences in elution seen for these peaks in Figure 8 represent variability between HPLC runs; the β-II peptide eluted one fraction earlier than the β-I peak in Figure 8A, and one fraction later in Figure 8C. Thus, the modifications which are responsible for the selective trypsin resistance of β-II, and presumably account for the difference in apparent molecular weight seen on SDS gels, appear to reside on peptide 123–133.

Changes in ionic charge can produce disproportionate shifts in the migration of proteins on SDS gel electrophoresis (Leach et al., 1980; DePaoli-Roach et al., 1983). To determine if the conversion of β-I to β-II involved a charged group, [3H]proline-labeled peptide 123–133 was collected from tryptic digests

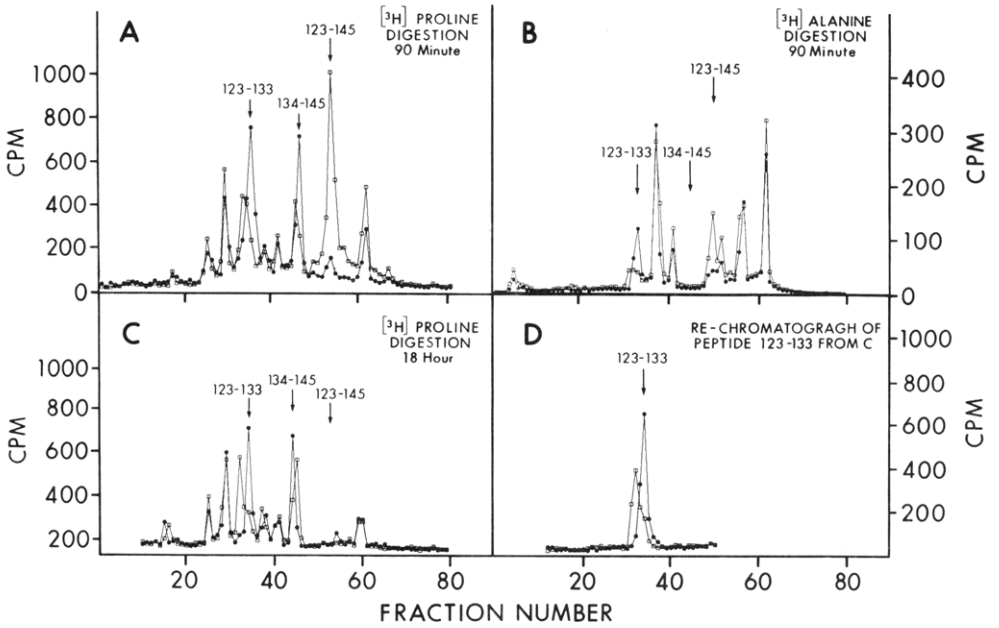

FIGURE 8: Tryptic digestion of β-I and β-II labeled with [3H]proline and [3H]alanine. β-I and β-II were immunoprecipitated from translations incubated at 27 and 37 °C, respectively, digested with trypsin for the indicated times, and analyzed by reverse-phase HPLC as described in the legend to Figure 7: (A) 90-min digestion of [3H]proline-labeled β-I and β-II; (B) 90-min digestion of [3H]alanine-labeled proteins; (C) 18-h digestion of [3H]proline-labeled proteins; (D) rechromatograph of peptides collected in fractions 31–35 of panel C. (●) β-I; (□) β-II. Arrows indicate the elution positions of synthetic peptide standards identical in sequence with hCG β carboxy-terminal residues 123–133, 134–145, and 123–145.

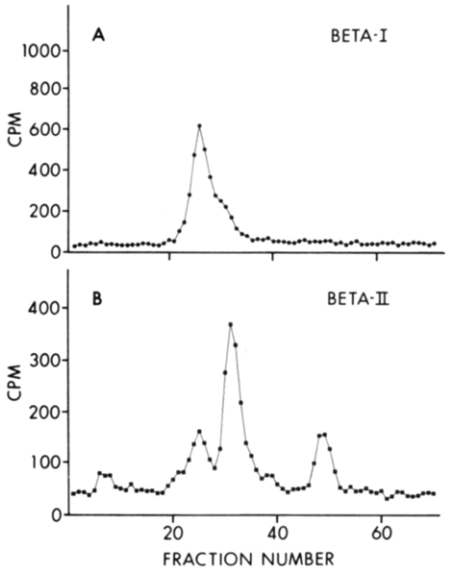

FIGURE 9: Anion-exchange HPLC of peptide 123–133 of β-I and β-II. [^{3}H]Proline-labeled peptides collected in fractions 31–35 of the reverse-phase HPLC runs shown in Figure 8C were loaded onto an Altex Ultrasil-AX column equilibrated with 22 mM sodium phosphate (pH 6.5). After a 10-min wash in the same buffer, the column was eluted with a 50-min linear gradient of 0–50% acetic acid in 22 mM sodium phosphate at a flow rate of 1 mL/min. One-milliliter fractions were collected. (A) β-I; (B) β-II.

of β-I and β-II and rechromatographed on anion-exchange HPLC. The β-I peptide eluted as a single peak on this column (Figure 9A), but β-II radioactivity was detected in three distinct peaks (Figure 9B). One peak coeluted with the β-I peptide at fraction 25. This material probably represents a small amount of β-I-derived unmodified peptide in the β-II preparation. Additional peaks at fractions 32 and 48 were also present in the β-II sample. These presumably are modified forms of the peptide. The presence of two modified peaks is consistent with the heterogeneity observed in the conversion reaction (note the intermediate bands seen on SDS electrophoresis in Figure 5) and may indicate that more than one amino acid of this peptide is modified. These peaks eluted significantly later in the gradient than the unmodified peptide, indicating that they may contain negatively charged groups, such as phosphate or sialic acid.

Figure 10 summarizes the results of the tryptic fingerprinting data. The modification responsible for the posttranslational conversion of β-I to β-II occurs at least in part on peptide 123–133 and probably affects an amino acid located sufficiently near the carboxyl terminus of this peptide to interfere with the accessibility of trypsin to arginine-133. It is noteworthy that we have localized this modification to a tryptic peptide (123–133) that contains two of the four serines which are known to be glycosylated in vivo (Birken & Canfield, 1977; Keutmann & Williams, 1977).

Further Characterization of β-II. To examine the possibility that serine-linked glycosylation might be occurring in this system, we digested β-II with neuraminidase and α-*N*-acetyl-D-galactosamine oligosaccharidase, an enzyme that cleaves asialated oligosaccharides which are O linked to serine residues (Pomato & Aminoff, 1978; Huang & Aminoff, 1972). β-II was found to be resistant this treatment (not shown). In addition, alkaline borohydride treatment (β-elimination), which releases O-linked oligosaccharides and reduces the linkage serine residues to alanine (Baenziger & Kornfeld, 1974; Downs & Pigman, 1976), failed to specifically generate [^{3}H]alanine from [^{3}H]serine-labeled β-I. These results imply that the production of β-II from β-I is not due to the addition of *N*-acetylgalactosamine to serine residues.

The ATP requirement of the reaction, as well as the acidic shift of the modified peptide on anion-exchange HPLC, suggested that the modification may involve a phosphorylation reaction. However, several incubations in which [α-^{32}P]ATP or [γ-^{32}P]ATP was included in the reaction mixture failed to label β-II. Furthermore, β-II was completely insensitive to digestion by phosphoprotein phosphatase (not shown). Thus, it appears unlikely that a simple phosphorylation reaction is responsible for the conversion of β-I to β-II.

Discussion

This study demonstrates that the primary translation product of hCG β mRNA undergoes a posttranslational modification during synthesis in ascites lysates. This modification is temperature dependent and is specific for the β subunit of hCG. Several lines of evidence indicate that the site of the modification is the carboxy-terminal region of hCG β. First, no modification was observed for LH β, which shares an 82% amino acid homology with hCG β but lacks the carboxy-terminal extension (Pierce & Parsons, 1981). Second, arginine-133 of β-II was found to be selectively resistant to limited tryptic digestion (Figure 10). A similar result was observed during studies of the trypsin sensitivity of mature hCG β, which contains carbohydrate on serine-132 (Birken et al., 1982). Third, complete tryptic digestion of β-II generated a modified fragment corresponding to carboxy-terminal peptide 123–133.

The chemical nature of this modification has proven to be difficult to identify. The resistance of neuraminidase-treated β-II to digestion by α-*N*-acetyl-D-galactosamine oligosaccharidase, an enzyme that cleaves asialated O-linked oligosaccharides (Pomato & Aminoff, 1978; Huang & Aminoff, 1972), implies that the modification is not due to addition of O-linked carbohydrate. Insensitivity to alkaline β-elimination further demonstrates the absence of serine-linked oligosaccharides on β-II.

The retention of modified peptide 123–133 on anion-exchange HPLC indicates that the modifying groups may be negatively charged. This result, in addition to the ATP requirement of the reaction, suggested that phosphate may be present on β-II. However, attempts to label β-II with [α-^{32}P]ATP or [γ-^{32}P]ATP were unsuccessful. Furthermore, β-II was found to be resistant to digestion by phosphoprotein phosphatase. Thus, it is unlikely that the modification involves a simple serine–phosphate linkage. These data cannot rule

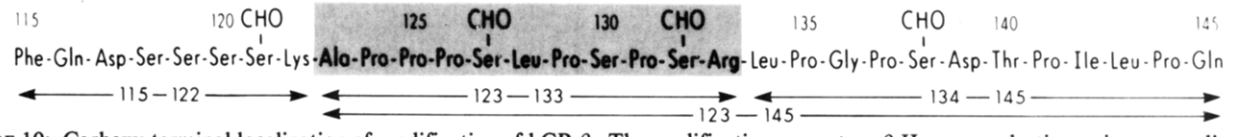

FIGURE 10: Carboxy-terminal localization of modification of hCB β. The modification present on β-II causes selective resistance to digestion by trypsin at arginine-133, producing peptide 123–145 in limited digestions (Figures 7 and 8A,B). Complete tryptic digestion of β-II produces a peptide corresponding to residues 123–133 which elutes earlier than the unmodified peptide on reverse-phase HPLC (Figure 8C,D) and is retained on anion-exchange HPLC (Figure 9). These results demonstrate that modifications of β-II reside on carboxy-terminal peptide 123–133. CHO indicates serine residues which contain carbohydrate in mature hCG β.

out the possibility that β-II contains phosphate in a protected linkage, however. Results of an extensive number of additional experiments have failed to detect other potential modifying groups, including sulfation, methylation, and ADP ribosylation.

The observation that efficient conversion of β-I to β-II was also produced by extracts of human placenta (Figure 6B) suggests that this modification may represent a normal step in the processing of hCG. The specificity of the reaction for the hCG β subunit, and its localization to a carboxy-terminal fragment which contains two serine-linked oligosaccharides in the secreted form of the subunit, suggests that our system is recognizing features of hCG β which allow it to be glycosylated in vivo.

Many protein sequences which serve as acceptors of O-linked oligosaccharides, including the carboxy-terminal region of hCG β, are rich in proline residues (Young et al., 1979). Some acceptors do not fit this pattern, however, and no consensus sequence similar to the Asn-X-Thr(Ser) triplet of asparagine-linked glycosylation has been identified (Schachter & Roseman, 1980). Presumably, the enzyme responsible for the initial transfer of carbohydrate to serine and threonine residues recognizes secondary structural features of the polypeptide. The modification of the carboxy-terminal region of hCG β observed here may represent an early event which facilitates recognition by an *N*-acetylgalactosamine transferase. The modification may exist only transiently and later be removed or supplanted by carbohydrate. It is noteworthy that no modification occurred on tryptic peptides 115–122 or 134–145, each of which contains one glycosylated serine residue in vivo, and that no change in migration on SDS electrophoresis was observed for the LH α subunit (Figure 2), which can serve as an acceptor of a threonine-linked oligosaccharide (Parsons et al., 1983). It will be interesting to determine if other known acceptors of O-linked oligosaccharides will serve as substrates in our system.

Hanover et al. (1982) have presented evidence that in BeWo choriocarcinoma cells incorporation of *N*-[^{3}H]acetylgalactosamine into the O-linked oligosaccharides of hCG β is a late event, occurring just prior to secretion. In contrast, the recent studies of Cummings et al. (1983) on the biosynthesis of the LDL receptor in fibroblasts indicate that galactosamine is added earlier in the secretory pathway, prior to the action of α-mannosidase I in the cis Golgi region. In either case, it is apparent that the specific O-glycosylation of hCG β is directed by its unusual carboxy-terminal segment and that reactions important for the generation of the structure of this region must occur quite early in the synthetic pathway. The temperature-dependent modification described here is specific for the hCG β subunit and may represent such an early step in the processing of its carboxy-terminal region.

Acknowledgments

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References

Andersson, G. N., & Eriksson, L. C. (1981) *J. Biol. Chem. 256*, 9633–9639.

Baenziger, J., & Kornfeld, S. (1974) *J. Biol. Chem. 249*, 7270–7281.

Baldwin, G. S., Knesel, J., & Monckton, J. M. (1983) *Nature (London) 301*, 435–437.

Bielinska, M., & Boime, I. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1768–1772.

Birken, S., & Canfield, R. E. (1977) *J. Biol. Chem. 252*, 5386–5392.

Birken, S., & Canfield, R. E. (1980) in *Chorionic Gonadotropin* (Segal, S. J., Ed.) pp 65–88, Plenum Press, New York.

Birken, S., Fetherston, J., Canfield, R., & Boime, I. (1981) *J. Biol. Chem. 256*, 1816–1823.

Birken, S., Canfield, R., Agosto, G., & Leis, J. (1982) *Endocrinology (Baltimore) 110*, 1555–1563.

Boime, I., Boothby, M., Hoshina, M., Daniels-McQueen, S., & Darnell, R. (1982) *Biol. Reprod. 26*, 73–91.

Chamberlain, J. P. (1979) *Anal. Biochem. 98*, 132–135.

Cole, L. A., Birken, S., Sutphen, S., Hussa, R. O., & Pattillo, R. A. (1982) *Endocrinology (Baltimore) 110*, 2198–2200.

Cummings, R. D., Kornfeld, S., Schneider, W. J., Hobgood, K. K., Tolleshaug, H., Brown, M. S., & Goldstein, J. L. (1983) *J. Biol. Chem. 258*, 15261–15273.

Daniels-McQueen, S., McWilliams, D., Birken, S., Canfield, R., Landefeld, T., & Boime, I. (1978) *J. Biol. Chem. 253*, 7109–7114.

DePaoli-Roach, A. A., Ahmud, Z., Camici, M., Lawrence, J. C., Jr., & Roach, P. J. (1983) *J. Biol. Chem. 258*, 10702–10709.

Downs, F., & Pigman, W. (1976) *Methods Carbohydr. Chem. 7*, 200–204.

Fetherston, J., & Boime, I. (1982) *J. Biol. Chem. 257*, 8143–8147.

Fiddes, J. C., & Goodman, H. M. (1980) *Nature (London) 286*, 684–687.

Glisin, V., Crkvenjakov, R., & Byus, C. (1974) *Biochemistry 13*, 2633–2637.

Godine, J. E., Chin, W. W., & Habener, J. F. (1982) *Biochem. Biophys. Res. Commun. 7104*, 463–473.

Goldberg, D. E., & Kornfeld, S. (1983) *J. Biol. Chem. 258*, 3159–3165.

Hanover, J. A., Lennarz, W. J., & Young, J. D. (1980) *J. Biol. Chem. 255*, 6713–6716.

Hanover, J. A., Elting, J., Mintz, G. R., & Lennarz, W. J. (1982) *J. Biol. Chem. 257*, 10172–10177.

Hortin, G., & Boime, I. (1981) *Cell (Cambridge, Mass.) 24*, 453–461.

Huang, C. C., & Aminoff, D. (1972) *J. Biol. Chem. 247*, 6737–6742.

Johnson, D. C., & Spear, P. G. (1983) *Cell (Cambridge, Mass.) 32*, 987–997.

Keller, D., Fetherston, J., & Boime, I. (1980) *Eur. J. Biochem. 108*, 367–372.

Keutmann, H. T., & Williams, R. M. (1977) *J. Biol. Chem. 252*, 5393–5397.

Kim, Y. S., Perdomo, J., & Nordberg, J. (1971) *J. Biol. Chem. 246*, 5466–5476.

Leach, B. S., Collawn, J. F., Jr., & Fish, W. W. (1980) *Biochemistry 19*, 5734–5741.

Matsuura, S., Chen, H.-C., & Hodgen, G. D. (1978) *Biochemistry 17*, 575–580.

Morgan, F. J., Birken, S., & Canfield, R. E. (1975) *J. Biol. Chem. 250*, 5247–5258.

Parsons, T. F., Bloomfield, G. A., & Pierce, J. G. (1983) *J. Biol. Chem. 258*, 240–244.

Pierce, J. G., & Parsons, T. F. (1981) *Annu. Rev. Biochem. 50*, 465–495.

Pomato, N., & Aminoff, D. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1602.

Ruddon, R. W., Hanson, C. A., Bryan, A. H., Putterman, G. J., White, E. L., Perini, F., Meade, K. S., & Aldenderfer,

P. H. (1980) *J. Biol. Chem. 255*, 1000–1007.
Ruddon, R. W., Bryan, A. H., Hanson, C. A., Perini, F., Ceccorulli, L. M., & Peters, B. P. (1981) *J. Biol. Chem. 256*, 5189–5196.
Ruddon, R. W., Cole, L. A., Pattillo, R. A., & Hussa, R. O. (1983) Abstracts of the 65th Endocrine Society Meeting, San Antonio, TX, June 1983, Abstr. 173.
Schachter, H., & Roseman, S. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., Ed.) pp 85–160, Plenum Press, New York.
Strous, G. J. A. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2694–2698.
Swarup, G., Cohen, S., & Garbers, D. L. (1981) *J. Biol. Chem. 256*, 8197–8201.
Szczesna, E., & Boime, I. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1179–1183.
Young, J. D., Tsuchiya, D., Sandlin, D. E., & Holroyde, M. J. (1979) *Biochemistry 18*, 4444–4448.

In Vitro Binding of Synthetic Acylated Lipid-Associating Peptides to High-Density Lipoproteins: Effect of Hydrophobicity†

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ABSTRACT: To measure the effect of hydrophobicity on the binding of model apoproteins to lipoproteins, we synthesized a 15 amino acid lipid-associating peptide (LAP) with acyl chains of various lengths (0–18 carbons) bound to the N-terminal amino acid through a peptide bond. The acylated LAPs preferentially bound to high-density lipoprotein (HDL) and were activators of lecithin:cholesterol acyltransferase. Circular dichroic spectra indicated that the LAP association with phospholipid was accompanied by increased α-helical structure. The LAPs self-associated in solution as judged from tryptophan fluorescence analysis. These characteristics, which are comparable to those of apolipoprotein A-I, were strongly dependent upon the acyl chain length of the LAPs. The equilibrium constants (K_{eq}) for the association of LAPs to reassembled HDL were measured by equilibrium dialysis at several temperatures. At 37 °C, K_{eq} increased by 3 orders of magnitude as the number of carbon units was increased from 0 to 16; there was a log-linear relationship between K_{eq} and the acyl chain length. The free energy of association (ΔG_a) decreased by a constant value for each methylene unit added to the acyl chain (0.35 kcal mol^{-1}), clearly demonstrating a strict hydrophobic effect. This change of ΔG_a was enthalpy rather than entropy driven. Our data show that, with all other parameters including putative α-helicity, sequence, and molecular weight being constant, the binding of a lipid-associating peptide to lipoprotein is governed by its hydrophobicity.

The plasma lipoproteins are water-soluble lipid–protein complexes that transport lipids in the circulation. They are operationally defined according to their densities as the high, low, intermediate, and very low density lipoproteins (HDL, LDL, IDL, and VLDL, respectively)[1] and chylomicrons. The lipoproteins are composed of a central core of nonpolar lipids, cholesteryl esters, and triglycerides surrounded by a monomolecular layer of polar lipids, unesterified cholesterol, phospholipids, and specific apoproteins (Schaefer et al., 1978; Smith et al., 1978). Most of the polar components spontaneously exchange among lipoproteins. The mechanism of transfer of apoproteins probably involves transport of monomers through the aqueous phase (Imaizume et al., 1978; Patsch et al., 1978; Pownall et al., 1978a, 1981). Analysis of the amino acid sequence of apolipoproteins led to the amphipathic helical theory of the lipid binding of apoproteins (Segrest et al., 1974). Hypothetically, when an apoprotein assumes a helical structure, the polar residues lie on one face of the helix, and the hydrophobic residues appear on the opposite side. Presumably, the nonpolar face of the helix penetrates the lipid matrix, and the polar face interacts with the aqueous phase. This theory has now been supported by numerous studies from many laboratories employing a variety of physicochemical techniques. When combined with a lipid matrix, apolipoproteins undergo changes in secondary structure consistent with formation of an amphipathic helix (Morrisett et al., 1973, 1977; Fukushima et al., 1980). Moreover, self-association of apoproteins in solution is one manifestation of their relatively high hydrophobicity (Stone & Reynolds, 1975; Mantulin et al., 1980; Massey et al., 1981a). Finally, the amphipathic helical theory has been tested with a number of model lipid binding peptides and with fragments of native apolipoproteins corresponding to lipid binding regions (Sparrow et al., 1973; Sigler et al., 1976; Mao et al., 1977; Chen et al., 1979; Fukushima et al., 1980; Kanellis et al., 1980; Pownall et al., 1980). These studies permitted the formulation of a number of properties of native or synthetic lipid-associating apoproteins: (i) the polypeptide must have the potential to form an am-

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[1] Abbreviations: HDL, high-density lipoprotein(s); LDL, low-density lipoprotein(s); IDL, intermediate-density lipoprotein(s); VLDL, very low density lipoprotein(s); LCAT, lecithin:cholesterol acyltransferase; POPC, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; apoA-I, apoprotein A-I; apoA-II, apoprotein A-II; C_n-LAP, lipid-associating peptide bearing an acyl chain of n carbons; R-HDL, reassembled HDL consisting of POPC and apoA-I (100:1); HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; CD, circular dichroism.

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